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## Effect of Immunization with Anti-Idiotypic Antibody to Melanoma Antigen on Lung Metastasis in Mice

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### Abstract

To determine whether the pulmonary metastases of melanoma cells could be inhibited, C57BL/6 mice were immunized with an anti-idiotypic antibody, 7C4, corresponding to a mouse melanoma antigen. Three groups of mice were compared: 1) 7C4-immunized group which received an injection of 100  $\mu$ g of 7C4 in Freund's complete adjuvant (FCA) subcutaneously on day -21 followed by intraperitoneal injections of the same dose on days -14 and -7, 2) adjuvant-treated control group administered with only FCA, and 3) untreated control group. On day 0,  $5 \times 10^5$  of BL6 cells were injected into the caudal vein of all mice. Two weeks later, they were sacrificed and their lungs were removed. The number pulmonary metastatic colonies present on the lung surface were counted and compared among the groups.

The length of survival days was also compared. The 7C4-immunized group showed an average of  $166 \pm 44$  colonies as compared to more than that of 300 colonies in each control groups, and a significant difference was observed ( $P < 0.01$ ). The immunized group survived significantly longer than the control group (Greenwood's formulation) on day 23 ( $P < 0.01$ ). Thus the immunization with 7C4 effectively inhibited lung metastasis of melanoma cells. These findings suggest that vaccination with anti-idiotypic antibody to tumor antigen is effective on inhibiting tumor metastasis.

### Introduction

Recently, many clinical trials of immunotherapy for cancer patients have accomplished, but, many investigators have not reported satisfactory result of immunotherapy. The major reason for unsatisfactory result owed that most of them were non-specific immunotherapies. In contrast, it is believed that tumor-specific immunotherapy is expected to be more efficient than non-specific im-

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Key word: Lung metastasis, Mouse melanoma, Anti-idiotypic antibody, Internal image antigen, Anti-idiotypic vaccination.

索引語: 肺転移, マウスメラノーマ, 抗イディオタイプ抗体, 内部抗原, 抗イディオタイプワクチン

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munotherapy<sup>1)</sup>. However, tumor antigens being essential to specific immunotherapy have been not always available whenever they were required.

More recently, monoclonal antibodies which are specific for antigens, have been able to be produced. Furthermore, anti-idiotypic antibodies, secondary antibodies, have been produced<sup>2-5)</sup>. The anti-idiotypic antibodies are considered to express the internal image antigens of original antigens, furthermore, they are stable for a long period<sup>6)</sup>. In other words, the antibodies as immunogens are ideal for specific immunotherapy.

RAYCHAUDHURI et al. have demonstrated that the immunization with the anti-idiotypic antibody inhibited the growth of primary subcutaneous tumor in mice<sup>7,8)</sup>. Thus, the anti-tumor effect of the anti-idiotypic antibody on the primary site have been reported, but, the anti-tumor effect on tumor metastasis have not been reported. If we would use the anti-idiotypic antibodies which were effective for the primary site, anti-tumor effect on tumor metastasis would be expected.

In the present study, we investigated whether immunization with an anti-idiotypic antibody could inhibit tumor metastasis.

### Materials and Methods

*Animals.* Female C57BL/6 mice were obtained from Nippon SLC, Shizuoka.

*Tumors.* Highly metastatic variant of mouse melanoma cell line B16-BL6 was established and provided by Dr. FIDLER<sup>9)</sup>.

*Anti-Idiotypic Antibodies.* The rat monoclonal antibody, RS-11, was obtained from syngeneic immunization with rat bladder tumor cells<sup>10)</sup>. RS-11 also reacted with a mouse bladder tumor cell line, BB-M1, and a mouse melanoma cell line, BL6.

Monoclonal anti-idiotypic antibodies were obtained by injecting BALB/c mice subcutaneously with 500  $\mu$ g of RS-11 in Freund's complete adjuvant (FCA) followed by the intraperitoneal injection of the same dose in Freund's incomplete adjuvant (FIA) twice at 7-day intervals. Then, the mice received an intravenous injection of the same amount of RS-11 7 days later. Spleen cells from the mice were prepared 3 days after the last injection and were hybridized with SP2/0 myeloma cells. Antibodies produced by the hybridomas were screened for binding specifically to RS-11 but not to normal rat Ig. Four clones (7C4, 9D4, 7C8 and 7C3) were obtained: The antibodies were identified as IgG<sub>1</sub> by immunodiffusion. In this report, an anti-idiotypic antibody 7C4 (Ab2) produced against antibody RS-11 (Ab1) was used as immunogen.

*Inhibition of RS-11 Binding to BB-M1 by 7C4.* BB-M1 cells ( $5 \times 10^5$ /ml) were cultured in a 96-well microplate (100  $\mu$ l/well) (CORNING, U.S.A.) for 24 h, and then fixed with acetone/methanol. The wells were filled with PBS containing 1% BSA for 1 h at room temperature. After rinsing with PBS, 50  $\mu$ l of serially diluted 7C4 was added to each well, and 50  $\mu$ l of biotinylated RS-11 (10  $\mu$ g/ml) was added 30 min after. The microplates were incubated for 1 h at room temperature. After washing, 50  $\mu$ l of avidin-biotinylated peroxidase complex (VECTASTAIN ABC KIT, U.S.A.) was added and incubated for 30 min at room temperature. After a final wash, 100  $\mu$ l of a chromogenic substrate (citrate buffer of pH 5 containing 0.4 mg/ml O-phenylenediamine and 0.02% H<sub>2</sub>O<sub>2</sub>) was added. After sufficient color had developed (~30 min), the reaction was terminated by adding 50  $\mu$ l of 2.5 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 492 nm on an enzyme linked immunosorbent assay (ELIZA) reader (Immuno Reader NJ-2000, Inter Med, Japan).

*Active Immunization.* C57BL/6 mice received a subcutaneous injection of 100  $\mu$ g of 7C4 in

FCA. 7 days later, they were injected intraperitoneally with the same amount in FIA twice at 7-day intervals. Then, 7 days after the last injection, antibody (Ab3) titer in the serum was measured as follows.

*Ab3 Titers in Sera of 7C4-Immunized Mice.* The serially diluted sera of mice immunized with 7C4 were added to the wells covered with BL6 monolayer and incubated for 1 h at room temperature. After washing, they were incubated with 50  $\mu$ l of 1/800 diluted HRP-conjugated sheep anti-mouse immunoglobulins (Amersham, U.K.) for 1 h at room temperature, washed, and developed with o-phenylenediamine (OPD).

*Measurement of Delayed-Type Hypersensitivity (DTH) Reaction.* All mice were primed subcutaneously with 5  $\mu$ g of anti-idiotypic antibody 7C4, and 2 weeks later,  $5 \times 10^5$  of BL6 cells of cell lysates were injected into the hind footpads, and thickness was measured 48 h later.

*Pulmonary Metastasis of BL6 Melanoma.* Mice were divided into three groups:

- ① 7C4-immunized group: This group was immunized with 7C4 in FCA and FIA as described above.
- ② Adjuvant-treated group: This group received injections of only Freund's adjuvant following the same schedule as the 7C4-immunized group.
- ③ Untreated control group: This group did not receive any treatment.

All mice had received an injection of  $5 \times 10^5$  BL6 cells into the tail vein at day 0. Then, the number of pulmonary metastatic colonies and the length of survival days were compared.

*Statistical Method.* The significance of the differences among the three groups was assessed by the Student's t-test.

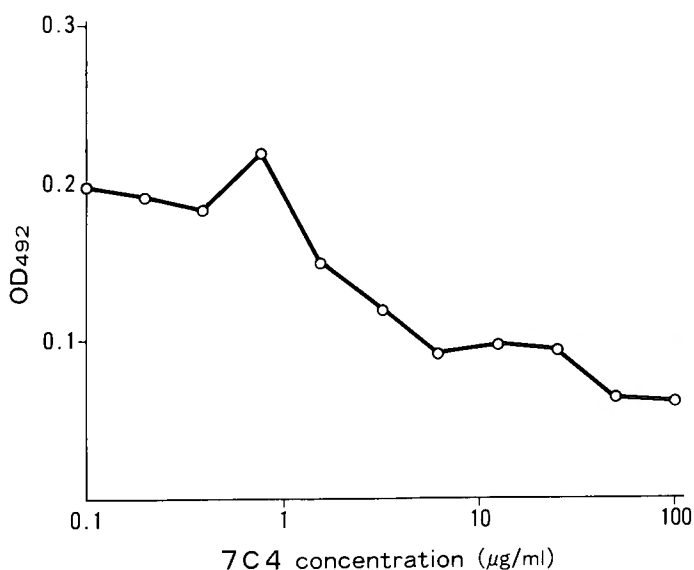


Fig. 1 Inhibition of RS-11 binding to BB-M1 by 7C4. Biotinylated RS-11 (10  $\mu$ g/ml) were added to the wells precoated with BB-M1 cells ( $5 \times 10^5$ /ml) in the presence of serially diluted 7C4, and its binding was developed by ABC and OPD. Each value indicates the means of triplicated wells.

## Results

*Inhibition of RS-11 Binding to BB-M1 by 7C4.* The anti-idiotypic antibody (Ab2), 7C4, was tested for its capability to block the binding of RS-11 (Ab1) to BB-M1 (Ag) (Fig. 1). The data demonstrated that the binding of RS-11 to BB-M1 could be efficiently inhibited by 7C4. This indicated the anti-ideotypic nature of 7C4 against RS-11.

*Ab3 Titers in Sera of 7C4-Immunized Mice.* Mice were immunized with 7C4 (Ab2), and their sera were assayed for the antibody activity against BL6 (Ag). Immediately after the completion of immunization, the serum antibody activity against BL6 was significantly higher in the 7C4-immunized group as compared with adjuvant and control groups ( $P < 0.01$ ) (Fig. 2). The titers continued to be significantly higher in this group on 14th day after the intravenous injection of BL6 cells ( $P < 0.01$ ) (Fig. 3).

*Inhibition of pulmonary metastasis of BL6.* The 7C4-immunized group ( $n=8$ ) showed an average of  $166 \pm 44$  colonies on the lung, whereas the control group ( $n=9$ ) showed more than that of 300 colonies, and also showed most of the adjuvant group ( $n=7$ ) (Fig. 4).

Metastasis of melanoma cells to the lung was significantly inhibited in the 7C4-immunized group as compared with the other two control groups ( $P < 0.01$ ) (Fig. 5). The 7C4-immunized group survived for the longest time (Fig. 6). By Greenwood's formulation, this group differed significantly from the control group on day 23 ( $P < 0.01$ ). The survival time of the adjuvant and control groups did not differ significantly.

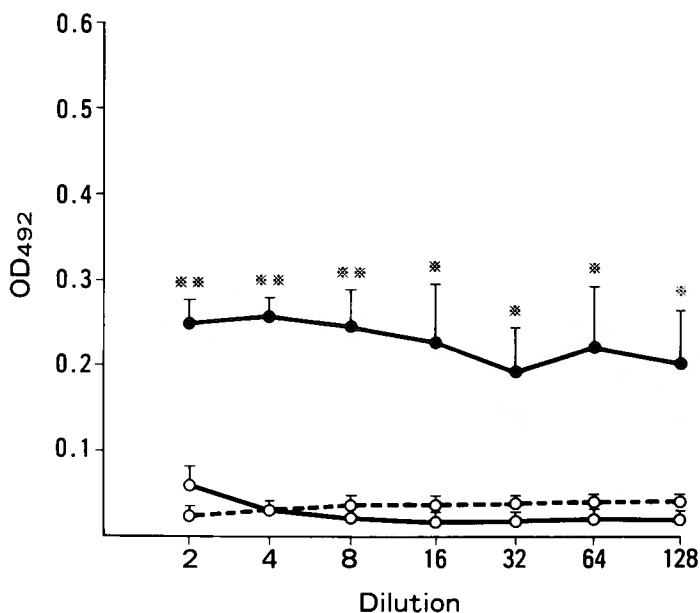


Fig. 2 Ab3 activity in the sera of 7C4-immunized mice immediately after the completion of immunization. Ab3 activity was assessed by their binding to plate coated with BL6 cells and developed by biotinylated anti-mouse Ig, ABC and OPD. Data are presented as mean + SD of triplicated wells. —●—: 7C4-immunized group ( $n=3$ ), ---○---: Adjuvant group ( $n=3$ ), —○—: Untreated group (control) ( $n=3$ ). \*:  $P < 0.05$ , \*\*:  $P < 0.01$ .

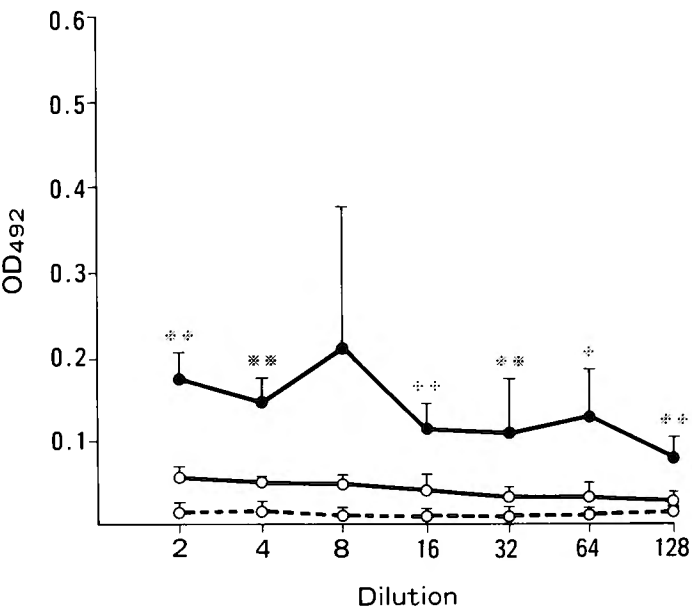


Fig. 3 Ab3 activity 2 weeks after the intravenous injection of BL6 cells. Data are presented as mean+SD of triplicated wells. —●—: 7C4-immunized group (n=5), ---○---: Adjuvant group (n=6), —○—: Untreated group (control) (n=4). \*: P<0.05, \*\*: P<0.01.

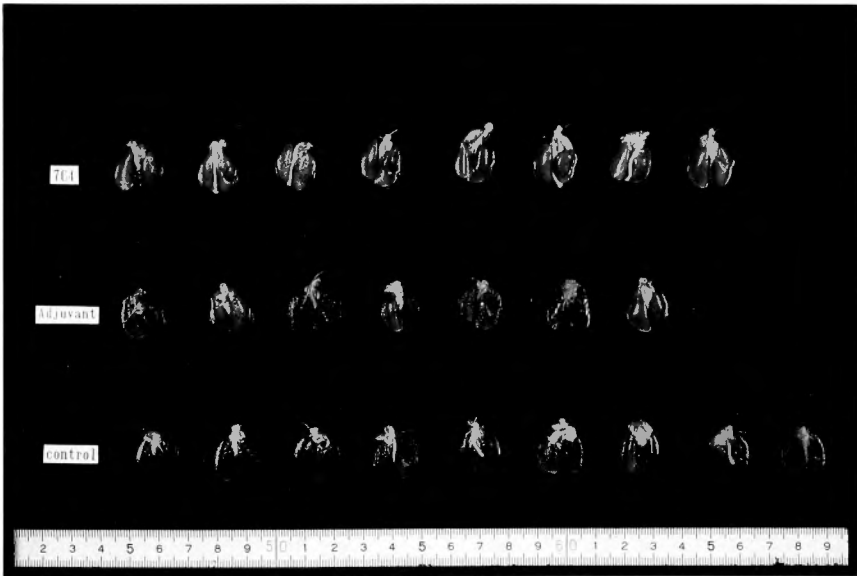


Fig. 4 Pulmonary metastases produced by BL6 melanoma cells 14 days after the intravenous injection.

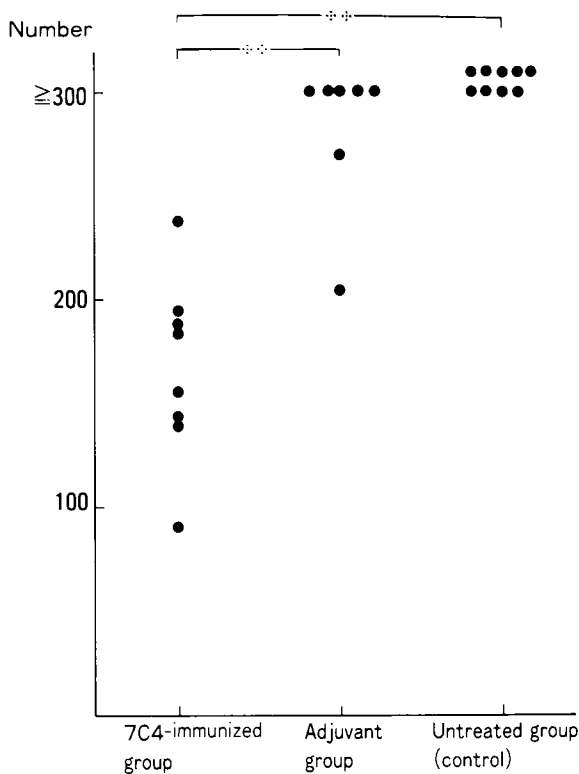


Fig. 5 Number of pulmonary colonies. \*\*:  $P < 0.01$ .

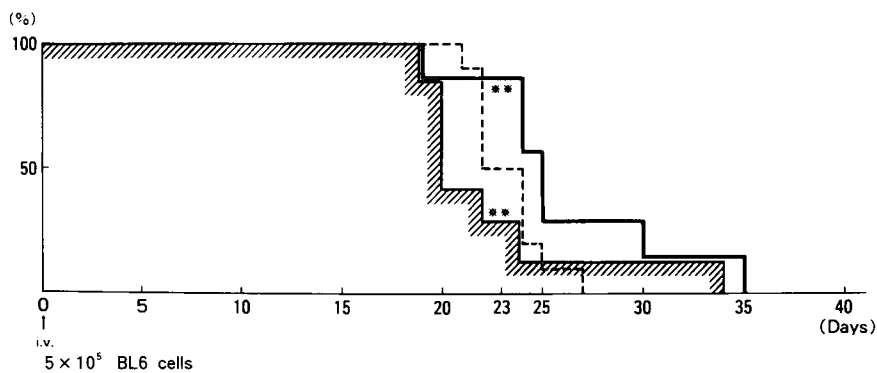


Fig. 6 Survival days following the intravenous injection of BL6 cells. —: 7C4-immunized group ( $n=7$ ), ---: Adjuvant group ( $n=10$ ), —: Untreated group (control) ( $n=7$ ). \*\*:  $P < 0.01$  (Greenwood's formulation).

*Priming of Delayed-Type Hypersensitivity by 7C4.* A significant swelling of footpads after challenging with BL6 cell lysates were observed in the 7C4-primed group as compared with the adjuvant and control groups ( $P < 0.01$ ) (Table 1).

Table 1 Delayed-type hypersensitivity reaction primed with 7C4

	Immunization	Challenge	Swelling (0.1 mm)
7C4-immunized group	7C4	BL6	2.8±0.50
Adjuvant group	FIA	BL6	0.9±1.11
Untreated group (control)	None	BL6	1.2±0.79

Footpad swelling was measured 48h after challenge of  $5 \times 10^5$  BL6 cells. \* :  $P < 0.05$ , \*\* :  $P < 0.01$ .

Data are presented as mean±SD.

FIA : Freund's incomplete adjuvant.

Discussion

Recently, many clinical trials of immunotherapy using biological response modifiers for cancer patients have accomplished. But, most of them were less efficient than they had been expected. One of the major reason for those unsatisfactory result is considered to be that most of them were so called "non-specific immunotherapies". If "tumor specific immunotherapy" were able to be performed, more efficient result would be expected. But this kind of therapies have been hampered by difficulties of getting the tumor specific antigens. More recently, anti-idiotypic antibodies have been able to be produced<sup>11-13)</sup>. The antibodies are considered to express the same structure of original antigens (internal image antigens)<sup>6)</sup>. RAYCHAUDHURI et al. have reported the anti-tumor effect of the anti-idiotypic antibody on the primary site<sup>7,8)</sup>. However, the effect of immunization with the anti-idiotypic antibody on tumor metastasis has not been examined.

In the present study, we investigated whether immunization with an anti-idiotypic antibody could inhibit tumor metastasis.

Ero et al. produced a rat IgM monoclonal antibody (Ab1), RS-11<sup>10)</sup>. It was confirmed that this antibody recognized a tumor-associated antigen (TAA) cross reactive among rats, mice and humans<sup>10)</sup>. Then, a secondary antibody (Ab2) to RS-11, 7C4, was induced by hybridization. Considering the cross reactivity of RS-11, 7C4 is thought to express the same internal image antigen of TAA of several tumor cells.

First of all, we examined whether 7C4 would express TAA of a mouse bladder tumor cell line, BB-M1. The result showed that 7C4 inhibited the binding of RS-11 to BB-M1. In short, it is considered that 7C4 inhibited the binding of RS-11 to BB-M1 since 7C4 bound RS-11 competitively. Therefore, 7C4 was found to express the internal image antigen of TAA of BB-M1.

RS-11 also reacted to a mouse melanoma cell line, BL6<sup>10)</sup>. So, we investigated whether immunization with 7C4 could induce third antibody (Ab3) to BL6 in mice. The elevation of antibody



titer to BL6 in murine sera was observed in the 7C4-immunized group. Thus, it was considered that 7C4 could induce Ab3 as the immunogen. The result showed that 7C4 expressed the internal image antigen of TAA of BL6.

On the basis of the fact, whether immunization with the anti-idiotypic antibody, 7C4, could inhibit tumor metastasis in mice was investigated. Lung metastasis model of BL6 was used.

The 7C4-immunized group showed an average of 166 metastatic colonies in the lungs, whereas non-immunized groups had that of over 300 colonies. The average of survival time was also investigated. A significant prolongation of survival time was observed in the 7C4-immunized group. Pathological study on dead mice of the non-treated group was proved that their lungs were filled with metastatic tumors. The findings suggested that the prolongation of survival time in the 7C4-immunized group owed to the inhibitory effect of 7C4 on lung metastasis. It was demonstrated that the immunization with the anti-idiotypic antibody inhibited the tumor growth of the primary site in mice<sup>7,8)</sup>. In our study, it was demonstrated that immunization with the anti-idiotypic antibody, 7C4, could inhibit tumor metastasis in vivo.

In general, it is thought that an anti-tumor effect by anti-idiotypic antibodies is dependent on antibody dependent cell mediated cytotoxicity (ADCC)<sup>14)</sup>.

Above mentioned, Ab3 titer in murine sera increased in the 7C4-immunized group. Ab3 titer continued to be significantly higher in the 7C4-immunized group than non-immunized groups for 14th days after the administration of BL6 cells. The findings indicated that Ab3 in the sera induced by 7C4 might play some role in inducing the anti-tumor effect by ADCC.

Furthermore, footpad swelling after 7C4 administration was measured to examine delayed-type hypersensitivity reaction. A significant footpad swelling was observed in the 7C4-primed group in comparison to adjuvant and control groups. These findings suggested that anti-idiotypic antibody, 7C4, might induce cell-mediated immunity. LEE et al. also reported that cell-mediated immunity could be induced against mouse bladder carcinoma by anti-idiotypic antibodies<sup>15)</sup>. But, to be certain this speculation, further investigation is required.

In this study, our present findings clearly demonstrated that the immunization with an anti-idiotypic antibody as an immunogen effectively inhibited tumor metastasis in mice. Immunotherapy using anti-idiotypic antibodies as an alternative tumor vaccine might be clinically useful in preventing human tumor growth and metastasis.

#### Acknowledgments

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## 和文抄録

マウスメラノーマの抗イデオタイプ抗体を用いた  
免疫感作による肺転移抑制効果の実験的検討

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マウスメラノーマ (BL6) の抗原に対応する抗イデオタイプ抗体 (7C4) を免疫原として感作を行ない, この免疫がメラノーマ細胞の肺転移に対して抑制的に働くかどうかを検討した。マウスは C57BL/6 マウスを用いた。実験は次の3群に分け行なった。① 7C4 免疫群: 免疫感作は, 7C4 100  $\mu$ g を BL6 細胞静注21日前に皮下注射, その7日後, 14日後に腹腔内注射して行なったもの。②アジュバント群: 同様にフロイントアジュバントだけを投与したもの。③対照群: 無処置のもの。これら3群に対して, BL6 細胞を  $5 \times 10^5$  個尾静脈より注入, 2週間後に肺を摘出し, 肺表面にみられる転移結節数を算定して比較した。アジュバント

群, 対照群では, ほとんど300個以上であった。一方, 7C4 免疫群では, 平均  $166 \pm 44$  個であり, 両群間に有意差が認められた ( $P < 0.01$ )。平均生存日数をみると, 7C4 免疫群で生存日数の延長が認められ, メラノーマ細胞静注23日目の時点で対照群に比べて有意差が認められた ( $P < 0.01$ )。これらの成績より, 抗イデオタイプ抗体 7C4 を用いた免疫がメラノーマ細胞の肺転移の抑制に対して有効であることがわかった。以上より, 腫瘍抗原の抗イデオタイプ抗体を用いたワクチン療法は, 癌転移の抑制に対して有効であることが示唆された。